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Research paper

The combination of TRAIL and MG-132 induces apoptosis in both TRAIL-sensitive and TRAIL-resistant human follicular lymphoma cells

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ABSTRACT

We have previously shown that the human follicular lymphoma cell line, HF28GFP, is sensitive to TRAIL-mediated apoptosis. Nevertheless, when the same cells overexpress anti-apoptotic Bcl-2 family protein, Bcl-xL (HF28Bcl-xL), they become resistant to TRAIL. Thus, these cell lines help us to investigate the action of novel apoptosis inducing candidate drugs. In the present study, we examined the effects of MG-132 (a proteasome inhibitor), LiCl (a glycogen synthase kinase-3 inhibitor) and/or TRAIL on pro-apoptotic Bcl-2 family proteins such as Bim and Bid. Here we demonstrate that the combination of MG-132 and TRAIL induced significant apoptotic cell death in both cell lines, HF28GFP and HF28BclxL. Apoptosis correlated with a decrease of phospho-ERK1/2, the accumulation of Bim and translocation of truncated Bid (tBid) and jBid. In addition, the combination of MG-132 and TRAIL seemed to target other apoptotic factors, which led to the accumulation of active capsase-3. Furthermore, co-stimulation of LiCl and TRAIL induced apoptosis in HF28GFP cells. However, HF28Bcl-xL cells were far less sensitive to the combinatorial effects of LiCl and TRAIL. Interestingly, we observed that LiCl did not target Bim and Bid proteins. In conclusion, these data show that targeting of pro-apoptotic Bcl-2 family proteins simultaneously through a selective proteasome inhibition might help to overcome TRAIL resistance caused by overexpression of anti-apoptotic Bcl-2 family proteins. Moreover, the data may provide new strategies to develop targeted therapies against lymphomas.

1. Introduction

Apoptosis is a type of programmed cell death that plays an essential role in the development and homeostasis of normal tissues. The two main apoptotic pathways are the death receptor pathway (extrinsic pathway) and the mitochondrial pathway (intrinsic pathway). These pathways are executed by caspases which eventually lead to the demise of a cell [1].

The death receptor signaling pathway is initiated by cytotoxic ligands such TRAIL (tumor necrosis factor-related apoptosis inducing ligand) and Fas. The activation of transmembrane TRAIL death receptors DR4 or DR5 by TRAIL leads to the formation of death-inducing signaling complex (DISC), where caspase-8 is activated. The active caspase-8 then cleaves and activates caspase-3, which results in apoptosis. Moreover, TRAIL selectively initiates apoptosis in a variety of tumor cells but not in normal cells. This special feature of TRAIL makes it a promising candidate for treatment of cancers [2,3].

Mitochondrial signaling pathway, however, is activated by a variety of non-receptor-mediated apoptotic stimuli such as cytotoxic drugs. This pathway is mainly regulated by Bcl-2 family proteins, a group of structurally related proteins. The Bcl-2 family proteins can be classified into three groups; 1) anti-apoptotic proteins such as Bcl-xL, Bcl-2, and Mcl-1, 2) multi-BH pro-apoptotic effector proteins, Bax and Bak, and 3) BH3-only proteins (pro-apoptotic sensitizers/activators) such as Bim and Bid [4].

The intrinsic apoptotic stimuli lead to the activation of pro-apoptotic Bcl-2 family proteins and/or down-regulation of anti-apoptotic proteins. For instance, up-regulation of Bim results in the activation of pore forming Bcl-2 family proteins, Bax/Bak either by 1) direct activation – the binding of Bim with Bax/Bak, or 2) indirect activation – freeing Bax/Bak from anti-apoptotic Bcl-2 family proteins. This phenomenon triggers mitochondrial outer membrane permeabilization (MOMP), thereby apoptotic proteins such as cytochrome c, and smac are released from the mitochondrial interspace into the cytosol. The

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formation of cytochrome c, Apaf-1 and ATP complex leads to the activation of caspase-9. Moreover, the released smac neutralizes the anti-apoptotic effect of XIAP (X-linked inhibitor of apoptosis protein). Consecutively, the activation of caspase-3 by caspase-9 leads to apoptosis [5,6].

As Bim is an important protein for the induction of apoptosis, resting cells regulate the turnover of this protein through phosphorylation and degradation by the survival kinase, ERK1/2. Inhibition of Bim degradation, therefore, can lead to cell death [7–9].

The cells which do not require the involvement of mitochondria in Fas/TRAIL-mediated apoptosis are called type I, whereas the cells that depend on the engagement of mitochondria are known as type II [10,11]. Bid has an important role in both type I and type II cells because caspase-8 mediated cleavage of Bid, truncated Bid (tBid – 15 kDa) connects the death receptors-induced signaling to mitochondria. tBid translocation into the mitochondria, thus, leads to the releases apoptotic factors. Moreover, it is interesting that JNK might induce caspase-8 independent cleavage of Bid at a distinct site to generate an intermediate product jBid (21 kDa) and the translocation of jBid to mitochondria preferentially releases smac to the cytosol [12].

XIAP protein suppresses apoptotic pathways. It binds and inactivates both caspase-9 and caspase-3. This special feature of XIAP enables it to prevent death receptor- and mitochondrial-mediated apoptosis. Furthermore, it has been shown that inhibition of XIAP switches type II signaling to type I signaling [13].

The proteasome is a large catalytic complex that is responsible for most non-lysosomal intracellular protein degradation of misfolded/damaged proteins. It also regulates protein turnover [14]. The proteasome, thus, regulates cellular homeostasis by maintaining the normal functions of cellular proteins. The proteasome, however, is also play an important role in the progression of cancer because it regulates critical proteins such as transcription factors. Therefore, proteasome inhibition is an attractive target for the development of anti-cancer therapies [15].

Proteasome inhibition can lead to cellular cytotoxicity through several mechanisms including, Bim accumulation by decreasing the amount of phospho-ERK1/2 [16], inhibition of NF-kB activity, and stabilization of p53. MG-132 (a proteasome inhibitor) is commonly used to investigate the proteasome activity in a range of cell types [17]. Moreover, MG-132 enhances TRAIL-induced apoptosis through up-regulation of DR5 in cancer cells [18].

Glycogen synthase kinase-3β (GSK3β) involved in multiple cellular processes including proliferation, glucose regulation and apoptosis [19]. Inhibition of GSK-3 by LiCl is found to augment the apoptotic effect of TRAIL in human lung carcinoma cells through up-regulation of death receptors DR4 and DR5 [20].

Overexpression of anti-apoptotic Bcl-2 family proteins prevents mitochondria-mediated apoptosis by blocking the permeabilization of outer mitochondrial membrane. We have previously shown that TRAIL induces apoptosis in HF28GFP cells (Type II cell model) but the apoptosis is completely prevented by overexpression of Bcl-xL (HF28Bcl-xL cells) [10]. In the present study, we examined the effects of apoptosis inducing agents: MG-132, LiCl, and/or TRAIL on Bid, Bim, and XIAP using HF28GFP cells (vector control) and HF28Bcl-xL cells.

2. Materials and methods

2.1. Cell lines and culture conditions

The origin and characteristics of human follicular lymphoma cell lines, HF28GFP and HF28Bcl-xL have been previously described [21]. The cells were cultured in RPMI 1640 medium (Lonza, Belgium) supplemented with 5% heat inactivated fetal bovine serum (GIBCO, Invitrogen, USA). 2 mM L-glutamine (Lonza), 106 U/ml streptomycin, 106 U/ml penicillin (Lonza), 10 mM HEPS buffer (Lonza), 0.1 mM nonessential amino acids (Lonza), 1 mM Na-pyruvate (Lonza), 20 μM 2-mercaptoethanol (Fluka-Chemie, Buch, Switzerland) at 37 °C in a 5% CO_2 humidified atmosphere.

2.2. Cell treatments

HF28GFP and HF28Bcl-xL cells were seeded on 6 or 12-well flat bottom polystyrene cell culture plates (Corning Inc. NY, USA) and treated with 50 ng/ml His-tagged recombinant human soluble killer TRAIL™ (Enzo life sciences, USA), 50 mM LiCl, (Sigma-Aldrich, USA) 1 μM MG-132 (Calbiochem, USA), 50 μM caspase-8 specific inhibitor, Z-IETD-FMK (Calbiochem, USA), 20 μM JNK specific inhibitor, SP600125 (Sigma-Aldrich, USA).

2.3. Flow cytometric analysis of apoptotic cells

Apoptotic cells were determined by flow cytometric analysis after propidium iodide (PI) staining. Cells with sub-G1 DNA content/hypodiploid cells were considered as apoptotic. Fixation and staining of cells were performed according to a standard protocol. In brief, at the end of stimulation times, samples containing one million cells were collected, resuspended in ice-cold PBS and fixed with ice-cold 70% V/V ethanol. After overnight incubation at +4 °C, cells were centrifuged at 1500 RPM for 10 min, resuspended in PBS containing 150 μg/ml RNAase (Sigma, USA) and incubated for 1 h at +50 °C. PI (Molecular Probes, Sigma) was added to the final concentration of 8 μg/ml and incubation was further continued 2 h at +37 °C. FACSCanto II flow cytometer with FACSDiva version 6.1.2 software (Becton Dickinson, USA) and FlowJo v10 software were used for the analysis.

2.4. Detection of changes in mitochondrial membrane potential

Depolarization of mitochondrial membrane was detected by TMRM (methyl ester of tetramethylrhodamine) staining. After incubation of cells with stimuli, 5×10^5 cells were collected and stained with 100 nM TMRM (Molecular Probes) for 20 min at +37 °C in the dark. After staining, the cells were immediately analyzed using a FACSCanto II flow cytometer (Becton Dickinson). The fluorescence excitation and emission maxima are 548 nm and 574 nm, respectively. The forward and side scatters were used to gate living or early apoptotic cells.

2.5. Preparation of total cell lysates

At the end of stimulation times, 2×10^6 cells were collected, washed in phosphate buffered saline (PBS) and centrifuged (600 × *g*, 5 min). The cell pellet was resuspended with lysis buffer containing 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 3% NP-40, 100 mM NaCl, 50 mM NaF, 1 mM PMSF, 1 mM VO_4, 5 μg/ml aprotinin, and 5 μg/ml Leupeptin. After 1 h incubation on ice, samples were centrifuged (10 000 × *g*, 15 min, + 4 °C). The protein concentration of the lysates was measured, equalized with sodium dodecylsulfate- polyacrylamide gel electrophoresis (SDS-PAGE) buffer (0.125 M Tris-Hcl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, bromophenol blue) and boiled for 5 min.

2.6. Preparation of mitochondrial and cytosolic fractions

Mitochondrial and cytosolic fractions were separated by an Apoalert cell fractionation kit (Clontech Laboratories, Inc., USA) according to the manufacturer's protocol. In brief, 2×10^7 cells were collected and washed with wash buffer. The cell pellet was resuspended in 0.8 mL fractionation buffer containing protease inhibitors and dithiothreitol (DTT) and incubated on ice for 10 min. Subsequently, cells were homogenized by passing the cell suspension through a 27G syringe needle. The homogenate was centrifuged (700 × *g*, 10 min, + 4 °C). The remaining supernatant was centrifuged (10 000 × *g*, 25 min, + 4 °C). The supernatant (cytosolic fraction) was collected and the pellet (mitochondrial fraction) was resuspended in 0.1 mL fractionation buffer.

2.7. Immunoblotting analysis

Equal amounts of protein were separated on a 12% or 15% SDS-PAGE gel and transferred to nitrocellulose membranes (GE Healthcare Life sciences, UK). Membranes were blocked with 1X TBS containing 3% BSA and 0.1% Tween-20 at room temperature. Then the membranes were incubated overnight at +4 °C with the primary antibodies (anti-caspase-8, anti-caspase-3, anti-Bid, anti-Bim, anti-phospho-ERK1/2, total ERK1/2, anti-phospho-BimEL (Ser77), anti-XIAP, anti-cytochrome c and anti-actin). Then the membranes were washed three times with 1X TBS and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Zymed laboratories Inc., USA) diluted in 3% BSA for 40 min at room temperature. The membranes were washed three times with 1X TBS. Finally, the membranes were developed for appropriate time period that yields the desired results using enhanced chemiluminescence detection system (GE Healthcare). Immunoblotting was done in duplicates.

2.8. Densitometry

The films were scanned and relative protein amounts were quantified using Quantity One Software. Immunoblotting results were normalized by actin values for each sample. A change in protein band intensities of the treated sample was compared to the control sample. Differences in protein band intensities were calculated and shown in fold.

2.9. Cell count

Live and dead cells were counted using hemocytometer. The cells were diluted 1:5 and stained with Erythrocin B. The dead cells/apoptotic cells stained pink, whereas the live cells remain unstained.

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism5. The statistical difference between groups was determined with student's *t*-test. P values < 0.05 were considered statistically significant.

3. Results

3.1. The combination of TRAIL with MG-132 or LiCl induces apoptosis in HF28GFP cells

It has been shown that MG-132 or LiCl enhances TRAIL-mediated apoptosis [18,20]. In this study, we examined whether the combination of TRAIL with MG-132/LiCl could enhance apoptosis in HF28GFP cells. Cells were cultured in medium containing LiCl, MG-132 and/or TRAIL. After incubation cells were collected for analysis. Mitochondrial depolarization and Sub-G1 DNA content were measured by TMRM and PI staining, respectively. The proportion of cells with collapsed mitochondrial membrane potential were 5% (LiCl), 20% (MG-132), 45% (TRAIL), 75% (LiCl + TRAIL) and 65% (MG-132 + TRAIL) (Fig. 1A). In addition, the proportion of apoptotic cells was 3% (LiCl), 10% (MG-132), 40% (TRAIL), 70% (TRAIL + LiCl) and 60% (TRAIL + MG-132) (Fig. 1B). MG-132 enhanced TRAIL-induced apoptosis (TRAIL vs MG-132 + TRAIL) ($P < 0.01$). Moreover, the live cells were counted. The proportion of viable HF28GFP cells after the treatment of TRAIL, TRAIL + LiCl, TRAIL + MG-132 were 68%, 38%, and 47%, respectively. The proportion of viable HF28Bcl-xL cells after the treatment of TRAIL + LiCl, and TRAIL + MG-132 were 94% and 71%, respectively (Fig. 2A). In addition, cell viability was analyzed by flow cytometry after PI staining. The viable cells were discriminated from apoptotic cells by analyzing dot-blot forward scatter (FSC) vs PI/PE (Fig. 2B). Cytochrome c release from the mitochondria and capase-3 activation were measured by immunoblotting from cytosolic extracts. MG-132 or TRAIL induced cytochrome c (cyt c) release to the cytosol and they also activated caspase-3. Moreover, the release of cyt c and caspase-3 activation was higher in the combination of TRAIL and MG-132 (Fig. 3A and B). Similarly, MG-132 or TRAIL induced the activation of caspase-8 and the level of active caspase-8 was increased when MG-132 or LiCl was combined with TRAIL (Fig. 3B).

3.2. The combination of MG-132 and TRAIL sensitizes HF28Bcl-xL cells to apoptosis

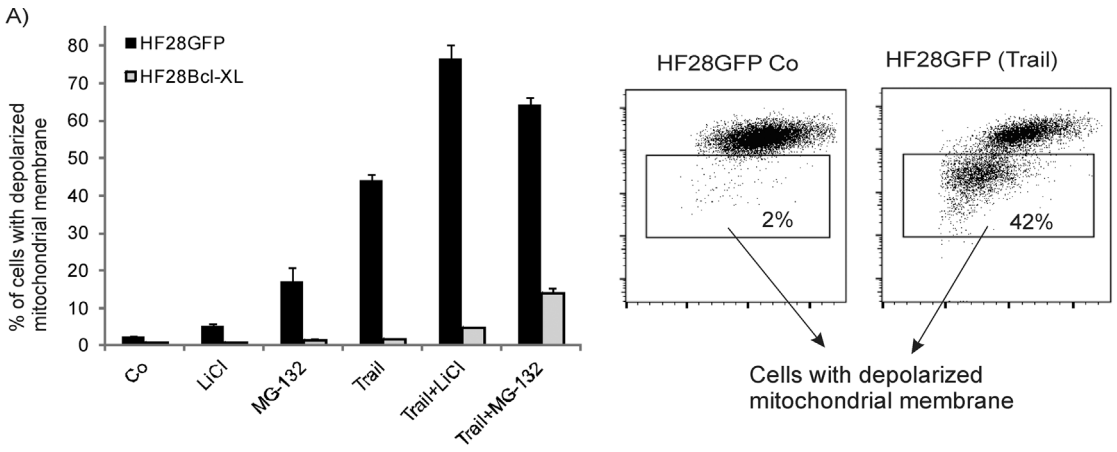
The combination of TRAIL with LiCl/MG-132 resulted in synergistic apoptosis in HF28GFP cells. Therefore, we further examined their apoptotic effects in TRAIL resistant HF28Bcl-xL cells. Cells were cultured in medium containing LiCl, MG-132 and/or TRAIL for 8 h. Mitochondrial depolarization and Sub-G1DNA content were analyzed. The proportion of cells with collapsed mitochondrial membrane potential was 4% (LiCl + TRAIL) and 15% (MG-132 + TRAIL) (Fig. 1A). Moreover, consistent with the membrane potential status, the combination of TRAIL and LiCl induced apoptosis (10% cell death) whereas, the combination of TRAIL and MG-132 resulted in significant apoptosis as the proportion of apoptotic cells was 35% (TRAIL vs TRAIL + MG-132) ($p < 0.001$) (Fig. 1B). In order to determine whether the small proportion of apoptotic cells observed in LiCl + TRAIL treated cells were due to a delayed response, the cells were incubated for 24 h. We found that an extended stimulation of cells increased cell death slightly, as the proportion of apoptotic cells increased from 10% (LiCl + TRAIL 8 h) (Fig. 1B) to 15% (LiCl + TRAIL 24 h) (Fig. 2C). However, the combination of MG-132 and TRAIL increased the proportion of apoptotic cells from 35% (8 h stimulation) (Fig. 1B) to 55% (24 h stimulation) (Fig. 2C). Furthermore, the combination of LiCl with TRAIL induced caspase-8 cleavage but not p12 caspase-3 (Fig. 3C). In addition, TRAIL alone induced the activation of caspase-8 but this led to an intermediate caspase-3 cleavage product, p20. Nevertheless, the active form of caspase-3, p17 and p12 were observed when MG-132 was combined with TRAIL (Fig. 3C).

3.3. Caspase-8 has no role in MG-132-induced Bid cleavage

Co-stimulation of MG-132 and TRAIL resulted in apoptosis in HF28Bcl-xL cells partly through mitochondria. Therefore, we investigated their effects on Bcl-2 family proteins such as Bid and Bim. Cells were incubated with MG-132 and/or TRAIL. After incubation cells were collected, proteins were isolated and immunoblotting was performed. Interstingly, stimulation of cells with MG-132 resulted in the cleavage of full length Bid into an intermediated Bid product (jBid) (Fig. 4B), whereas TRAIL, as expected produced truncated bid (tBid) in both cell types (Fig. 4A and B). Moreover, to examine whether caspase-8 was involved in the production of jBid, cells were incubated with caspase-8 specific inhibitor, Z-IETD for 1 h prior to the addition of MG-132 for 8 h. Furthermore, in order to ensure that Z-IETD-FMK was actively functioning, HF28GFP cells were treated with the inhibitor 1 h prior to the addition of TRAIL for 4 h. Z-IETD-FMK completely prevented TRAIL-induced apoptosis in HF28GFP cells (vector control) (Fig. 4D), whereas the inhibitor did not prevent the production of jBid (Fig. 4C). In addition, JNK has been linked with production of jBid, however, in HF28Bcl-xL cells a JNK-specific inhibitor, SP600125, led to cell death (Data not shown).

3.4. MG-132 leads to the accumulation of Bim

We and others have previously shown that ERK is an important player in mediating phosphorylation of Bim to confer cell survival [7,22]. Moreover, inhibition of Bim degradation by MG-132 sensitizes cancer cells to cell death [8]. Therefore, we examined whether MG-132 prevents ERK-mediated degradation of Bim or decreases phospho-ERK1/2. The amount of Bim was analyzed by immunoblotting. The



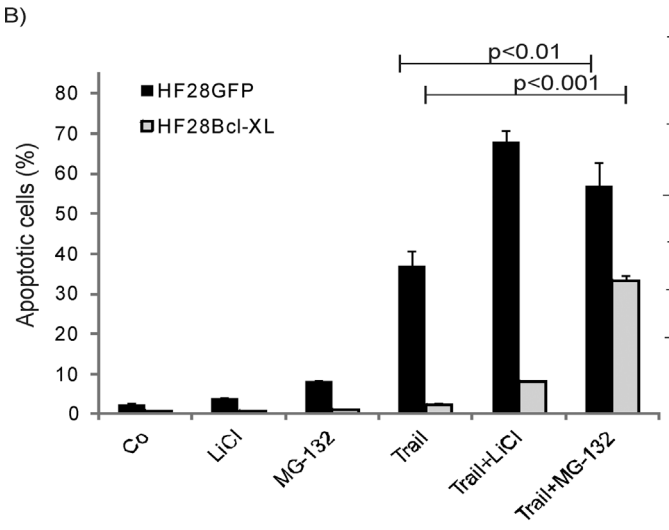


Table 1

DNA content (%)

Cell cycle stages	Co	TRAIL	TRAIL + LiCl	TRAIL + MG-132
(Apoptotic) Sub-G1	1	4	8	33
G0/G1	53	52	42	33
S	29	29	34	22
G2/M	17	15	16	12

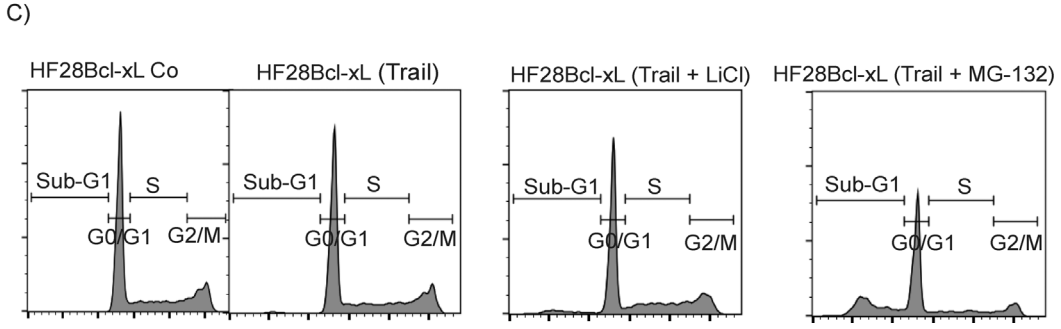


Fig. 1. The effects of LiCl, MG-132 and/or TRAIL-mediated apoptosis in human follicular lymphoma cells. Vector control HF28 cells (green fluorescent protein [GFP]) and HF28 cells overexpressing Bcl-xL were treated with the indicated apoptotic stimuli for 8 h. After incubation cells were collected for analysis. (A) Mitochondrial membrane potential collapse was measured by flow cytometry using TMRM. The TMRM analysis of control (Co) and TRAIL is presented. The same gating strategy was used in all TMRM analysis. (B) Sub-G1 DNA contents were analyzed by flow cytometry after PI staining. Data are presented as mean ± SEM from three independent experiments. The statistical significance of difference was determined using student's *t*-test. (C) Cell cycle analysis for control cells and cells treated with TRAIL, TRAIL + LiCl, and TRAIL + MG-132 is presented. The stages of cell cycle: Sub-G1, G0/G1, S, G2/M are indicated in the histogram. The proportion of DNA content of cells at different stages of cell cycle is shown above the histogram (Table 1). The same gating strategy was used in all PI analysis.

amount of BimEL protein decreased in control cells, whereas MG-132 led to the accumulation of BimEL in both cell lines (Fig. 4E). Moreover, the expression of phospho-ERK1/2, total ERK1/2 and phospho-Bim (Ser77) were analyzed (Fig. 4E). MG-132 significantly decreased the level of phospho-ERK1/2 without affecting the total amount of ERK1/2 (Fig. 4E). Moreover, MG-132 decreased the amount of downstream target protein, phospho-BimEL (Ser77). However, LiCl did not result in accumulation of Bim (data not shown).

3.5. Down-regulation of XIAP does not act as switch between type I and type II TRAIL-induced apoptosis

It has been shown that XIAP regulates the switch from type II to type I signaling in Fas-induced apoptosis [23]. We, therefore, investigated the effect of LiCl, MG-132 and/or TRAIL on XIAP protein level in HF28Bcl-xL cells. Cells were stimulated with these apoptotic stimuli. After stimulation cells were collected, proteins were isolated and

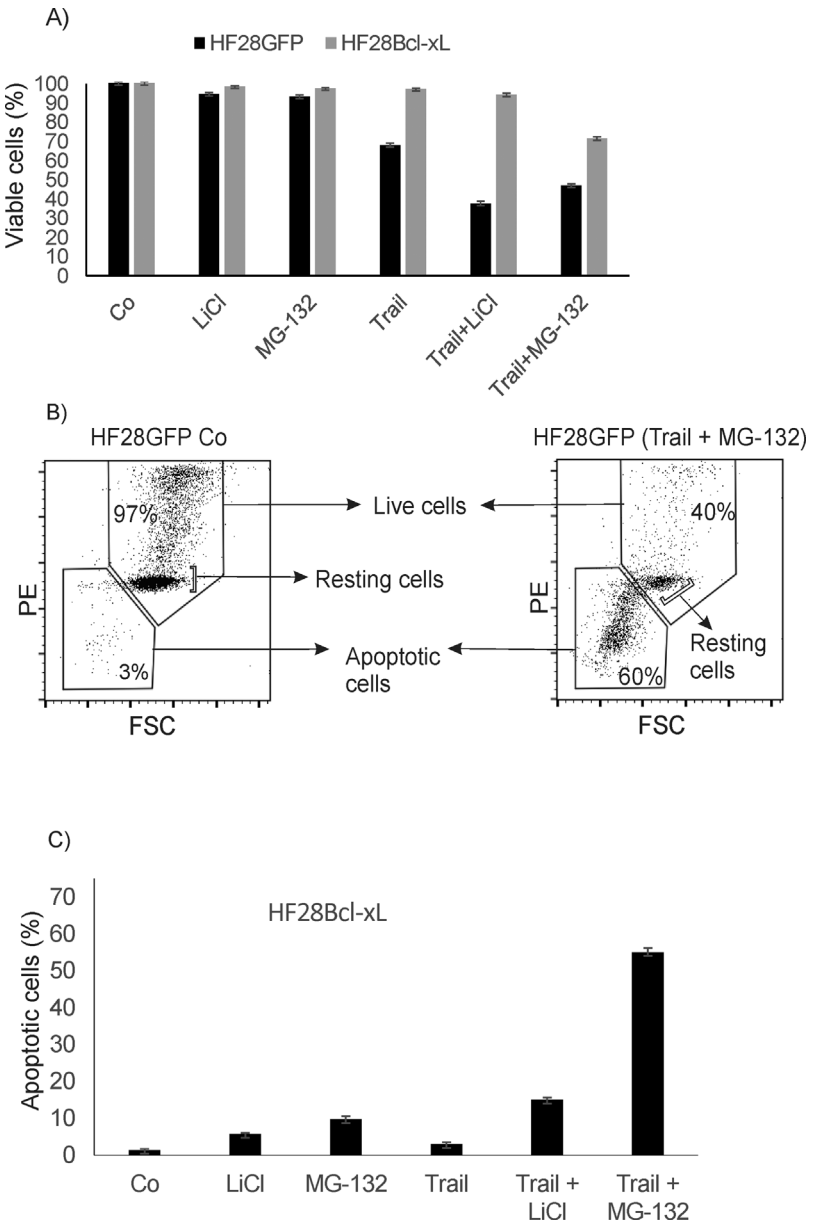


Fig. 2. Viable cells count. (A) The cells were incubated with LiCl, MG-132, TRAIL, TRAIL + LiCl, and TRAIL + MG-132 for 8 h. At the end of stimulation time, cells were collected and stained with Erythrocin B and counted using hemocytometer. Data are presented as mean ± SEM from three independent experiments. (B) Live cells and apoptotic cells were analyzed by flow cytometry. PE vs FSC gating strategy was used to discriminate the cells. (C) HF28Bcl-xL cells were incubated with LiCl, MG-132, TRAIL, TRAIL + LiCl, and TRAIL + MG-132 for 24 h. Sub-G1 DNA contents were analyzed by flow cytometry after PI staining. Data are presented as mean ± SEM from three independent experiments.

immunoblotting was performed. Interestingly, the combination of LiCl and TRAIL led to a significant down-regulation of XIAP amount (the relative amount in fold compared to the control was 0.35) (Fig. 3D), however, HF28Bcl cells were far less sensitive to apoptosis was not induced (Fig. 1B). In addition, MG-132 in combination with TRAIL slightly decrease the amount of XIAP (the relative amount in fold compared to the control was 0.85) (Fig. 3D).

4. Discussion

In the present study, we demonstrate that MG-132 or LiCl enhanced TRAIL-mediated apoptosis in HF28GFP cells. However, in contrast to LiCl, MG-132 strongly sensitized HF28Bcl-xL cells to TRAIL because the proportion of LiCl- and TRAIL-induced apoptotic cells was 3.5 times less than the proportion of apoptotic cells after MG-132 and TRAIL treatment (Fig. 1Figs. 1B and Fig. 22C). Therefore, we further investigated the role of MG-132 in apoptosis. MG-132 is a proteasome inhibitor and can target a variety of molecular pathways. However, we mainly focused on its effect on XIAP and pro-apoptotic Bcl-2 family proteins (Bid and Bim). We choose these proteins because of their critical importance in mitochondrial-mediated apoptosis (Fig. 5).

MG-132 produced an intermediate cleavage product of full length Bid, jBid, in HF28Bcl-xL cells. In addition, MG-132 decreased the level of phospho-ERK1/2, thereby preventing degradation of BimEL (Fig. 4E). These findings show that MG-132 has broader range of effects than simply inhibiting the proteasome pathway. Consistent with our findings, it has been shown that MG-132 reduces the amount of phospho-ERK1/2 [24]. Furthermore, TRAIL induced tBid. Therefore, the combination of MG-132 and TRAIL led to the translocation of jBid, tBid and the accumulation of Bim. As a result, mitochondria were depolarized. The translocation of either jBid or tBid, however, was not enough to depolarize the mitochondria or induce apoptosis.

It is interesting that we observed jBid only in HF28Bcl-xL cells but not in HF28GFP cells. These results indicate that when mitochondria were fully protected by overexpressed Bcl-xL, MG-132 might activate an apoptotic pathway which resulted in the production of jBid. JNK is a key regulator of many cellular events including apoptosis [25]. As a pro-apoptotic kinase, it is linked to the production of jBid. However, JNK specific inhibitor, SP600125, accelerated apoptosis (data not shown) in HF28Bcl-xL cells indicating that JNK has a pro-survival role

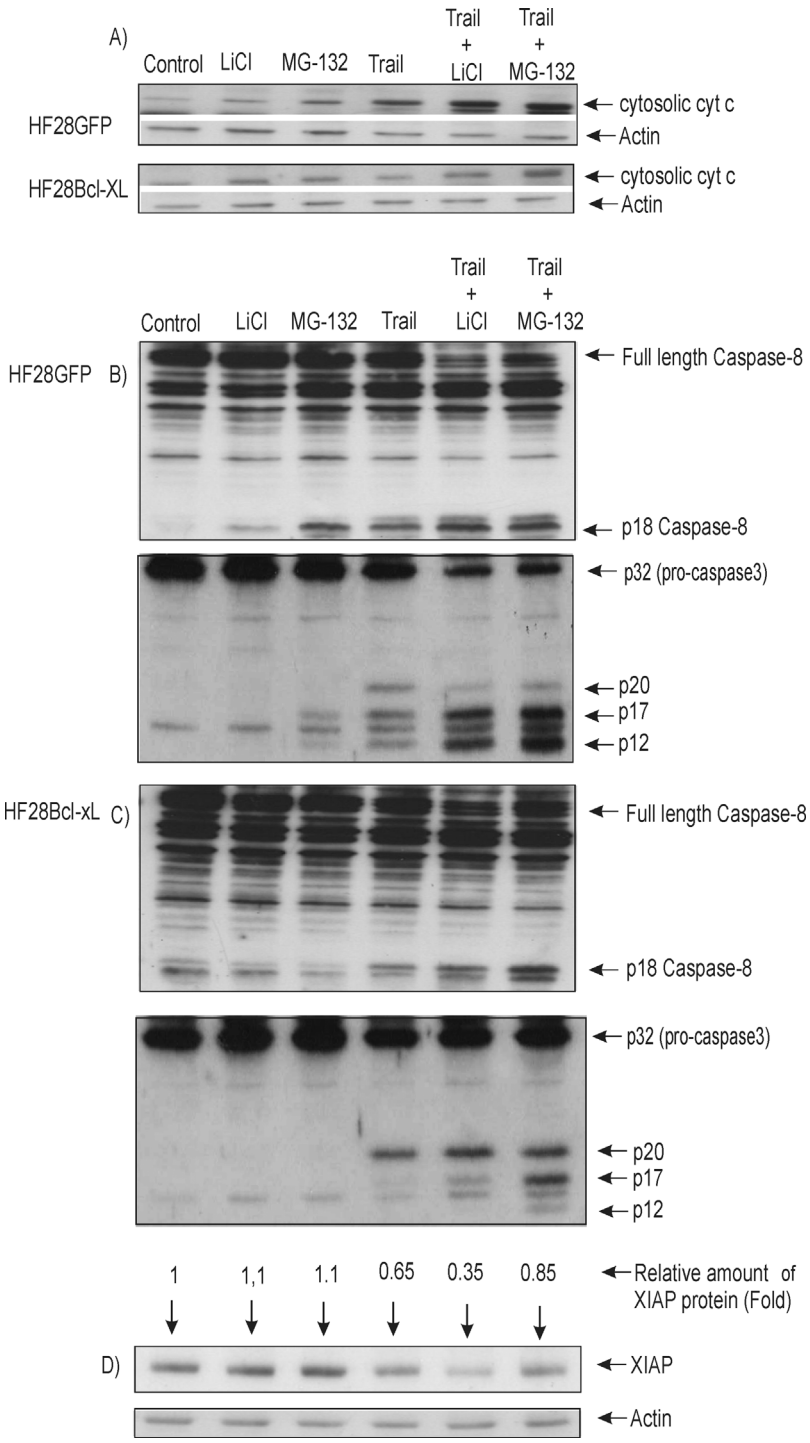


Fig. 3. The effects of LiCl, MG-132 and/or TRAIL on cytochrome c release, caspase activation and XIAP protein level. The mitochondrial and cytosolic proteins were separated as described in "Materials and methods". (A) Cytochrome c release in HF28GFP and HF28Bcl-xL cells was detected by immunoblotting. (B) Activation of caspase-8 and caspase-3 in HF28GFP cells was analyzed by immunoblotting. (C) Activation of caspase-8 and caspase-3 in HF28Bcl-xL cells was analyzed by immunoblotting. (D) XIAP protein level in HF28Bcl-xL cells were detected by immunoblotting. Moreover, XIAP protein level of control, and cells treated with LiCl, MG-132, TRAIL, TRAIL + LiCl and TRAIL + MG-132 was quantified. Antibody against actin was used to ensure that equal amount of protein was loaded on the gel. These samples were analyzed from the same experiments which were used for TMRM and PI analysis.

in HF28 cells. Furthermore, caspase-8 has no role in producing jBid, as caspase-8 specific inhibitor Z-IETD-FMK did not prevent the production of jBid.

Moreover, MG-132 induced active form of caspase-8 (p18) was only seen in HF28GFP but not in HF28Bcl-xL. It is well-known that caspase-8 can be activated either by death receptor stimulation or caspase-3 [26]. Therefore, based on our findings, we suggest that the activation of caspase-8 in HF28GFP cells was mediated by active caspase-3. Firstly, we did not observe MG-132-mediated activation of caspase-3/caspase-8 in HF28Bcl-xL cells. Thus, caspase-3 to caspase-8 activation feedback loop was missing. Secondly, as MG-132 is not a cognate ligand to death receptors (TRAIL-R and Fas-R), it is unlikely that MG-132 leads to caspase-8 activation through these receptors.

In HF28GFP cells, the proportion of TRAIL-induced apoptotic cells and cells with depolarized membrane were 40% and 45%, respectively. Whereas in HF28Bcl-xL cells, the proportion of MG-132 + TRAIL-induced apoptotic cells and cells with depolarized membrane were 35% and 15%, respectively. These results show that the proportion HF28Bcl-xL cells with depolarized membrane was four times less than the proportion of HF28GFP cells with depolarized membrane. Nevertheless, the proportion of apoptotic cells between the two cell lines were comparable. Therefore, we suggest that MG-132- and TRAIL-mediated apoptosis was largely depend on type I signaling pathway. In addition, the results analyzed by viable cells count and the PI staining were also

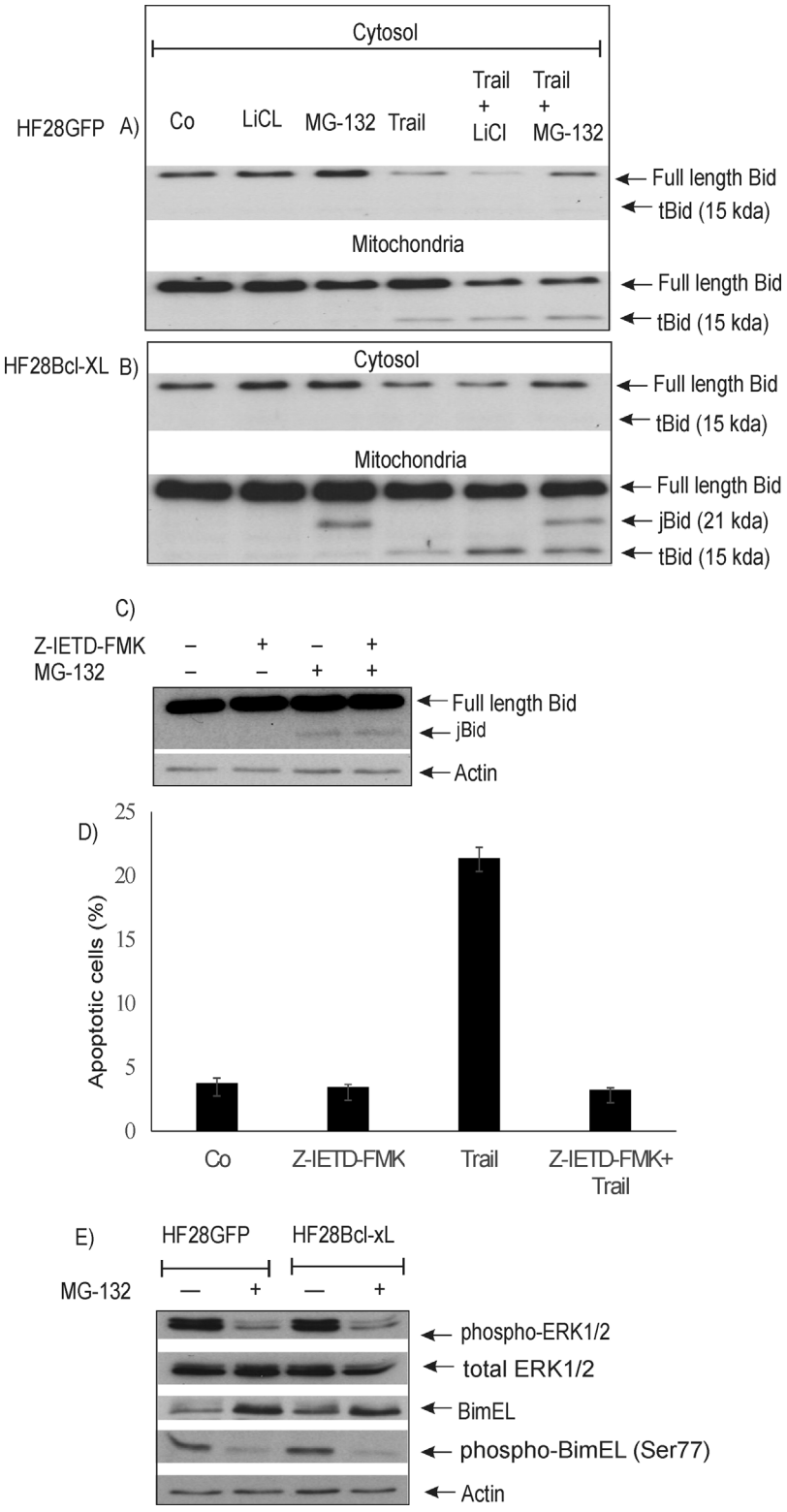


Fig. 4. MG-132 produces an intermediate Bid cleavage, jBid and/or an accumulation of Bim in HF28Bcl-xL/HF28GP cells. (A) Full length Bid and tBid were detected by immunoblotting from cytosolic/mitochondrial extracts of HF28GFP cells. (B) Full length, jBid and tBid were detected by immunoblotting from cytosolic/mitochondrial extracts of HF28Bcl-xL cells. These samples were analyzed from the same experiments which were used for TMRM and PI analysis. (C) HF28Bcl-xL cells were treated with caspase-8 specific inhibitor, Z-IETD-FMK for 1 h prior to the addition of MG-132 for 8 h. In addition, cells were also treated with Z-IETD-FMK for 1 h prior to the addition of TRAIL for 4 h. After the incubation cells were collected for analysis. Total cell lysates were prepared for immunoblotting. Full length Bid and jBid were detected by immunoblotting. Actin was also used. (D) Sub-G1 DNA contents of the cells were analyzed. (E) HF28GFP and HF28Bcl-xL cells were treated with MG-132 for 8 h. After incubation the cells were collected and total cell lysates were prepared. The levels of phospho-ERK1/2, total ERK1/2, Bim, phospho-BimEL were analyzed. Actin was also used.

comparable.

In mitochondrial-induced apoptosis, we always observed the active form of caspase-3, P17 [27,28]. However, in death receptor (TRAIL-induced apoptosis), the intermediate caspase-3 product, p20 is detected (Fig. 3B and C). This non-apoptotic caspase-3 product should further be processed to induce apoptosis. Interestingly, in the present study, we demonstrate that the combination of MG-132/LiCl with TRAIL induced a massive mitochondrial depolarization. As a result, a significant amount of p17 was produced, which led to cell death. Clearly, these data show that the apoptotic factors from mitochondria are required to eliminate the inhibitor of p20. We also show that the combination of MG-132 and TRAIL induced mitochondrial depolarization in HF28Bcl-xL cell. We, therefore, suggest that this event might contribute to the activation of p17.

It has been shown that inhibition of XIAP protein in mitochondria-dependent type II cells switches type II to type I death receptor

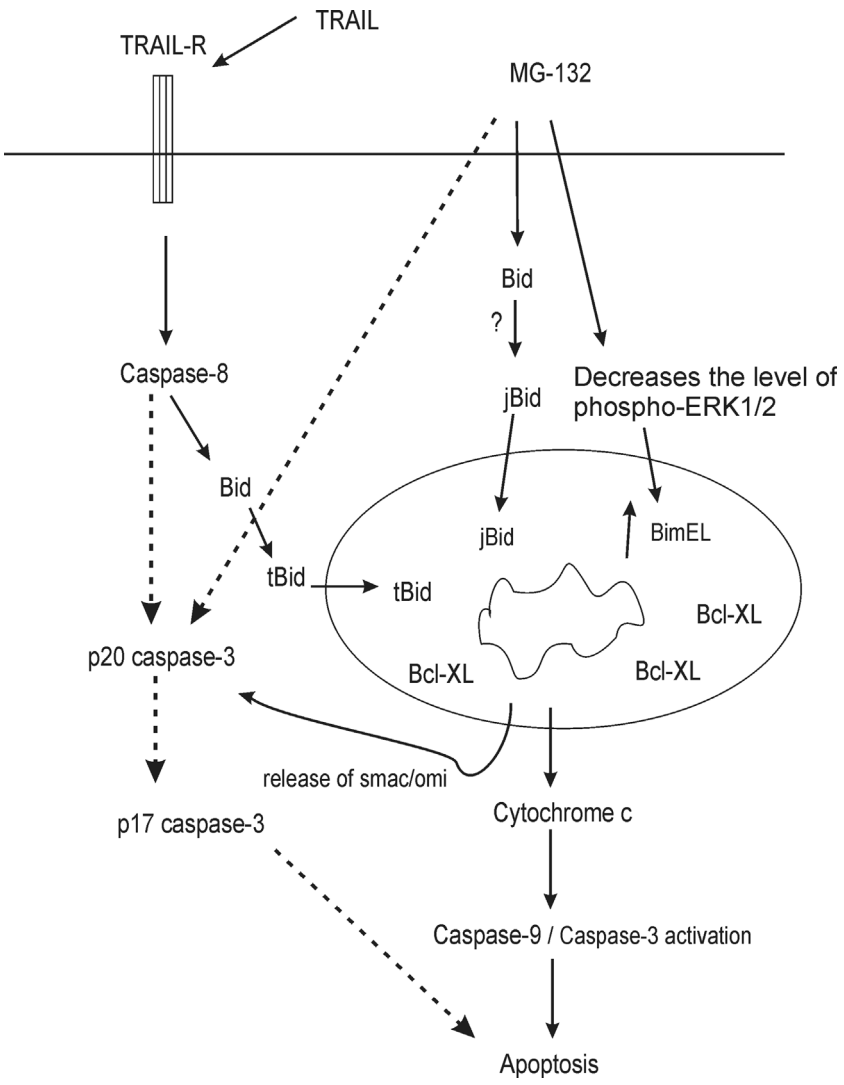


Fig. 5. Model of MG-132 and TRAIL-induced apoptosis in HF28Bcl-xL/HF28GFP cells. Activation of TRAIL receptor by TRAIL leads to the activation of caspase-8, which in turn cleaves Bid into tBid (15 kDa). This active Bid translocates into mitochondria. In addition, MG-132 cleaves Bid into jBid (21 kDa) in HF28Bcl-xL cells, which is also translocates into mitochondria. Furthermore, MG-132 decreases the amount of phospho-ERK1/2 and prevents degradation of BimEL. Therefore, the cumulative effects of tBid, jBid and accumulation of BimEL lead to mitochondrial depolarization and apoptosis. Moreover, apoptotic factors such as smac/omi can be released and neutralize inhibitor of caspase-3 processing. However, the combination of MG-132 and TRAIL induces mainly type I signaling pathway (see dashed lines).

signaling [23]. Moreover, XIAP is known to bind and prevent caspase-3 activation [29]. In this study, we show that the combination of TRAIL + LiCl significantly down-regulated XIAP amount. Nevertheless, these apoptotic stimuli failed to produce the active caspase-3 cleavage, p12. However, we speculate that a very low proportion of apoptotic HF28Bcl-xL cells which resulted from TRAIL + LiCl was due to the activation of low level of p17. These data indicate that XIAP is not a critical protein in switching type II to type I signaling in HF28 cells. Thus, a protein which bind to p20 remains to be investigated. However, it is important to mention here that caspase-3 could be regulated through post-translational modifications. For instance, cellular inhibitor of apoptosis (cIAP1) dependent ubiquitylation of active subunits of caspase-3, p12 and p17, leads to proteasome-dependent degradation of these effector caspases. These findings show that disabling the ubiquitin-proteasome pathway could induce apoptosis by stabilizing the active caspase-3 subunits [30]. Interestingly, in HF28GFP and HF28Bcl-xL cells, the combination of MG-132 and TRAIL led to the accumulation of active caspase-3 subunits, p12 and p17 while there was still a significant amount of an intermediate caspase-3 form, p20. It is, therefore, likely that MG-132 stabilized p12 and p17 from degradation and thereby enhancing apoptosis.

Bortezomib (PS-314) is a first-in-class proteasome inhibitor, which is used in clinics for the treatment of mantle cell lymphoma and multiple myeloma. In addition to its direct action, bortezomib enhances the standard chemotherapy or radiotherapy treatment. Moreover, a new class of proteasome inhibitors such as carfilzomib and marizomib have been developed to increase the efficiency of proteasome inhibition while decreasing its side-effects [31,32]. We, therefore, suggest that the combination of TRAIL and a highly selective proteasome inhibitor might be beneficial to treat lymphoma patients.

In conclusion, we demonstrate that the combination of MG-132 with TRAIL induces apoptosis in TRAIL-resistant HF28Bcl-xL cells partly through the accumulation of Bim and Bid cleavage products, jBid and tBid. In addition, it seems that the production of jBid is dependent on the membrane potential status of a cell because jBid is only produced when mitochondrial depolarization is fully prevented or the proportion of cells with depolarized membrane is minimal. Furthermore, given the specificity of TRAIL against cancer cells, the combination of TRAIL agonists with highly specific proteasome inhibitors might be an effective treatment strategy for patients with relapsed/TRAIL refractory lymphomas.

Conflict of interest statement

The authors declare no financial or commercial conflict of interest.

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